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Opposing Effects of Ras on p53: Transcriptional Activation of *mdm2* and Induction of p19^{ARF}

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Summary

Mdm2 acts as a major regulator of the tumor suppressor p53 by targeting its destruction. Here, we show that the *mdm2* gene is also regulated by the Ras-driven Raf/MEK/MAP kinase pathway, in a p53-independent manner. Mdm2 induced by activated Raf degrades p53 in the absence of the Mdm2 inhibitor p19^{ARF}. This regulatory pathway accounts for the observation that cells transformed by oncogenic Ras are more resistant to p53-dependent apoptosis following exposure to DNA damage. Activation of the Ras-induced Raf/MEK/MAP kinase may therefore play a key role in suppressing p53 during tumor development and treatment. In primary cells, Raf also activates the Mdm2 inhibitor p19^{ARF}. Levels of p53 are therefore determined by opposing effects of Raf-induced p19^{ARF} and Mdm2.

Introduction

Mdm2 was originally identified as an amplified gene in a spontaneously transformed derivative of BALB/c cell line 3T3 DM, which caused tumors when injected into nude mice (Fakharzadeh et al., 1991). A possible mechanism for the transforming properties of *mdm2* has been provided by reports demonstrating that Mdm2 is a major regulator of the tumor suppressor p53. It binds directly to p53 and inhibits its transcriptional activity (Momand et al., 1992; Oliner et al., 1992, 1993). Mdm2 is a transcriptional target of p53 (Barak et al., 1993; Wu et al., 1993; Leng et al., 1995). p53-responsive elements have been identified in the intronic promoter of the *mdm2* gene, and interaction of p53 with these sites has been well documented (Juven et al., 1993; Barak et al., 1994; Zauberman et al., 1995). Induction of *mdm2* transcription by p53 establishes a negative feedback loop, in which p53 itself initiates its own destruction (Picksley and Lane, 1993).

Mdm2 may have additional functions that are not directly related to p53. It affects cell growth in a p53-independent manner, possibly through interactions with pRb (Xiao et al., 1995) or through interaction with the E2F/DP1 complex (Martin et al., 1995). These functions of Mdm2 are less well characterized than its role in p53 regulation. Indeed, the lethal effects of disrupting *mdm2* genes in vivo are rescued by disrupting p53, suggesting that p53 regulation is the major function of this protein at least during early development (Jones et al., 1995; Montes de Oca Luna et al., 1995).

Mdm2 binds to p19^{ARF} and is inhibited by this interaction (Kamijo et al., 1998; Pomerantz et al., 1998). An attractive model has been presented recently in which p19^{ARF} binds to Mdm2, sequesters Mdm2 in nucleolar structures, and allows accumulation of p53 (Tao and Levine, 1999; Sherr and Weber, 2000). On the other hand, p19^{ARF} directly inhibits Mdm2 ubiquitin ligase activity, suggesting a more direct role in Mdm2 regulation (Honda and Yasuda, 1999). p14^{ARF}, the human homolog of p19^{ARF}, is induced by E2F, myc and Ras, and thus provides a possible link from mitogenic signaling pathways to p53 induction (Bates et al., 1998; Palmero et al., 1998; Zindy et al., 1998). The Ras-regulated Raf/MEK/ERK kinase pathway has been reported to activate CDK4/cyclin D kinases, thereby phosphorylating pRb, which in turn leads to release of E2F-1 (Albanese et al., 1995; Peeper et al., 1997). The p14^{ARF} promoter contains several E2F-1 binding sites and its activity was shown to be enhanced by overexpression of E2F-1 (Bates et al., 1998). The Ras/Raf/MEK/MAP kinase pathway can therefore lead indirectly to accumulation of p14^{ARF} and inhibition of Mdm2 activity.

Mdm2 expression is often increased following mitogenic activation. Treatment of cells in culture with basic FGF increases levels of Mdm2 protein and cells constitutively exposed to a basic FGF autocrine loop are more refractory to killing by cisplatin, which to a large extent occurs through p53-mediated apoptosis (Shaulian et al., 1997). More recently, a screen for transcripts that accumulate in cells harboring a chimeric M-CSF/PDGF receptor identified *mdm2* as an immediate early gene (Fambrough et al., 1999). In addition, we have observed high levels of Mdm2 protein expression in human tumor cell lines that have little, if any, functional p53 (Ries et al., 2000). These data suggest regulation of *mdm2* expression by p53-independent pathways triggered by growth factors.

A common feature of signaling by diverse growth factors is the activation of the Ras/Raf/MEK/MAP kinase pathway. We therefore explored the possibility that this pathway is responsible for *mdm2* induction. We report that the *mdm2* promoter is indeed a target of the Ras/Raf/MEK/MAP kinase pathway. Hence, activation of Ras during normal cell signaling or through mutation in neoplastic transformation, can suppress p53 and thus facilitate cell proliferation and survival.

Results

Mdm2 Expression Is Regulated by the Ras/Raf Pathway

Mdm2 protein can be induced by exposure of cells to basic FGF (Shaulian et al., 1997) or IGF-1 (Leri et al., 1999). Likewise, *mdm2* mRNA accumulates upon activation of the PDGF receptor (Fambrough et al., 1999).

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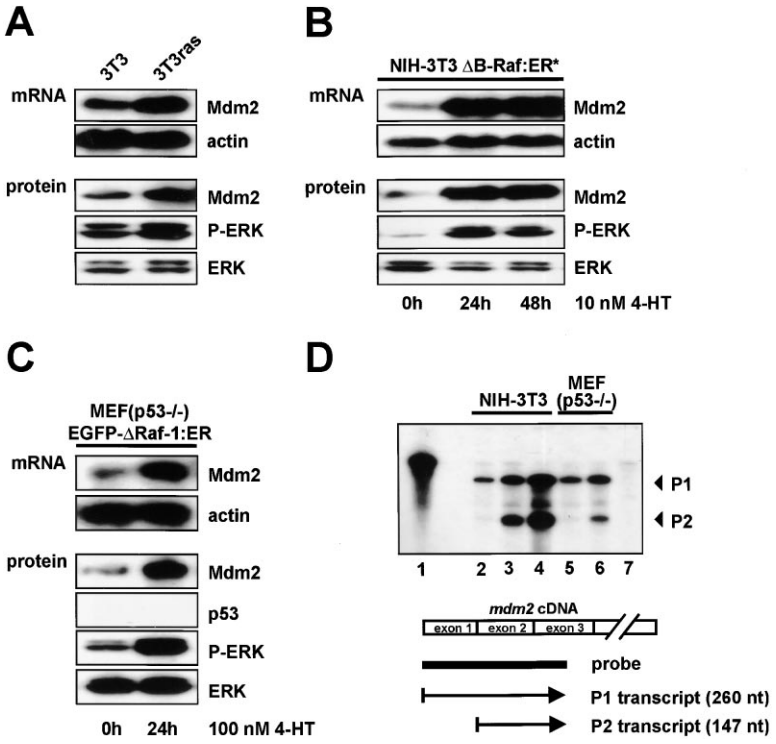

Figure 1. Induction of *mdm2* Transcription by Activated Ras and Raf

(A) *Mdm2* transcriptional induction by constitutively active H-Ras(G12V) in NIH-3T3 cells. NIH-3T3 cells were infected with retroviruses encoding H-Ras(G12V) or empty vector. After selection with G418, total cellular RNA was prepared and subjected to Northern blot analysis using a 1.5 kb fragment spanning the coding region of *mdm2* as a probe. For Western blot analysis, cells were directly lysed with Laemmli buffer, and equal amounts of total protein were subjected to immunoblotting.

(B) Activation of Raf is sufficient to induce *mdm2* transcription in NIH-3T3 cells. NIH-3T3 cells expressing a 4-Hydroxy-Tamoxifen (4-HT) inducible ΔB-Raf:ER* construct were treated with 10 nM 4-HT, total cellular RNA and protein harvested at the times indicated and subjected to Northern blot analysis or Western blot analysis.

(C) Transcriptional induction of *mdm2* by Raf is independent of p53. $p53^{-/-}$ mouse embryonic fibroblasts (MEF) harboring the 4-HT inducible EGFP-ΔRaf1:ER construct were treated with 100 nM 4-HT, and total cellular RNA and protein was harvested after 24 hr.

(D) Transcription by activated Raf initiates mainly from the internal *mdm2* promoter (P2). Total cellular RNA was prepared from the cell lines as indicated (top). Ten micrograms of each RNA was subjected to RNase protection analysis. Lanes 1 and 7 contain the probe alone and the control reaction consisting of 10 μg of yeast tRNA, respectively. Composition and sizes (in nucleotides) of potential RNase-resistant fragments are indicated in the schematic representation.

Since Ras is downstream of all these mitogens, we tested whether the Ras pathway is responsible for *mdm2* induction. NIH-3T3 cells expressing an activated Ras allele, H-Ras (G12V), were analyzed for *mdm2* mRNA and protein expression. Figure 1A shows that expression of constitutively active Ras resulted in increased levels of *mdm2* mRNA and protein.

Ras targets several distinct downstream effectors, of which the best characterized are Raf kinase, PI3-kinase, and RalGDS (Marshall, 1995; Katz and McCormick, 1997; Rodriguez-Viciana et al., 1997). We examined the effects of a conditionally active Raf kinase on cellular Mdm2 levels. This conditional Raf kinase consists of the kinase domain of B-Raf fused to a modified form of the hormone binding domain of the mouse estrogen receptor, which renders the receptor insensitive to estrogen, but still sensitive for the estrogen analog 4-hydroxy-tamoxifen (4-HT) (Samuels et al., 1993; Woods et al., 1997). NIH3T3 cells stably expressing the ΔB-Raf:ER* were treated with 10 nM 4-HT (Woods et al., 1997). Addition of 4-HT resulted in remarkable MAPK activation and accumulation of high levels of *mdm2* mRNA and protein (Figure 1B). To rule out any contribution of p53 to the increased Mdm2 levels after Raf activation, we assessed Mdm2 expression in $p53^{-/-}$ mouse embryo fibroblasts (MEFs) stably expressing EGFP-ΔRaf1:ER (Woods et al., 1997). Figure 1C shows increased transcription and accumulation of Mdm2 protein in response to Raf/MEK/MAPK activation occurred also in $p53^{-/-}$ fibroblasts. In a time course experiment using NIH3T3(ΔB-Raf:ER*) cells, elevated *mdm2* mRNA levels can be detected as early as 4 hr after Raf activation and increase steadily (data not shown). Thus, the Ras/Raf/MEK/MAP kinase pathway induces increased expression of *mdm2* mRNA and Mdm2 protein in a p53-independent manner.

The murine *mdm2* gene has two promoters, an internal promoter (P2), which responds directly to p53 activation, and an upstream constitutive promoter (P1), which is not affected by p53 (Barak et al., 1994). The cDNA riboprobe used for RNase protection assay spans exon 1, exon 2, and part of exon 3 (Figure 1D), allowing discrimination between P1 and P2 transcripts. Transcription from the P2 promoter was dramatically induced in response to activation of the Raf kinase, whereas a more moderate induction occurred with transcription from P1 (Figure 1D, lanes 2, 3, and 4). Induction of P2 transcription occurs in a p53-independent manner as p53-deficient MEFs show similar enhanced transcription arising from P2 (Figure 1D, lanes 5 and 6). Note that only P1 transcripts are detectable in control NIH-3T3 cells.

Analysis of Ras-Responsive Elements within the *mdm2* P2 Promoter

To determine the role of Raf activation on basal *mdm2* transcription, the effect of a constitutively activated form of Raf (Raf-CAAX) on *mdm2* promoter activity was examined in p53-deficient 10(1) cells (Stokoe et al., 1994). Raf-CAAX expression induced *mdm2* P2 promoter activity 5- to 6-fold (Figure 2B). Sequence analysis of the *mdm2* P2 promoter revealed the existence of binding sites for transcription factors of the AP-1 and Ets family (Figure 2A), which have been shown to be responsive to ERK activation in other genes. To determine the region of the *mdm2* P2 promoter required for regulation by Raf, several *mdm2* P2 5′ promoter deletions were generated and transfected in the presence of Raf-CAAX into 10(1) cells. Figure 2B shows that the upstream Ets site within the P2 promoter (designated EtsA in Figure 2B) of the *mdm2* gene is important for the Raf-induced

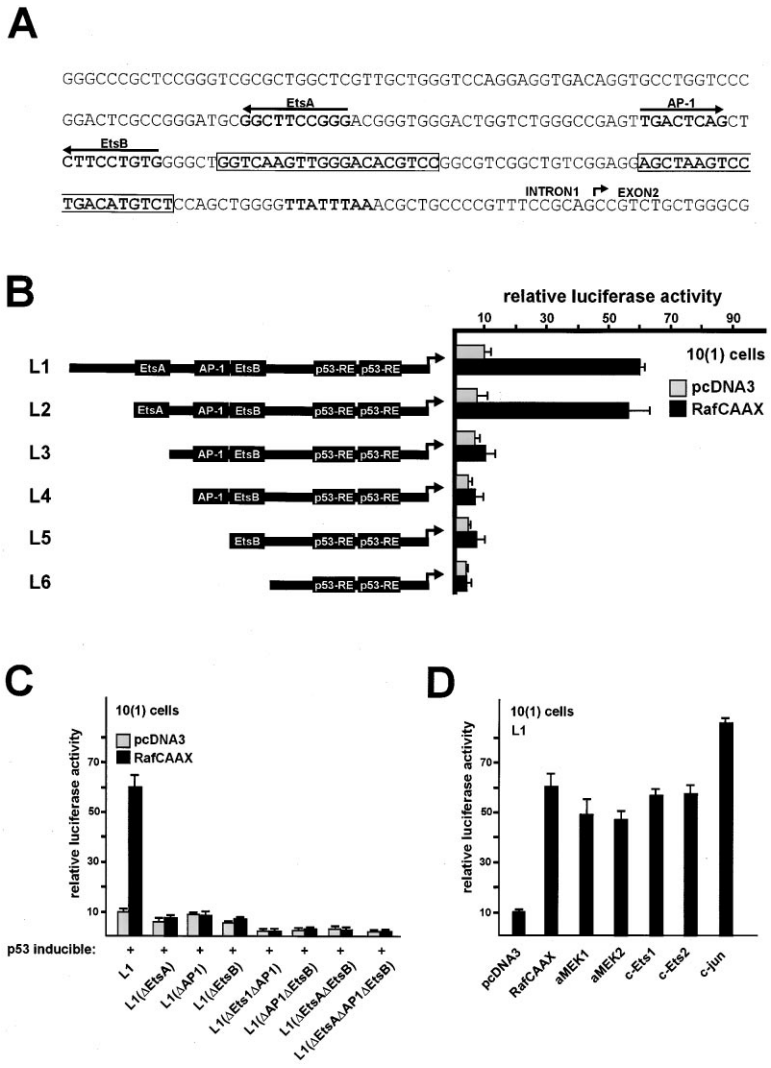

Figure 2. Ets/AP-1 Motifs in the Intronic P2 Promoter Mediate Transcription of the *mdm2* Gene upon Raf Activation

(A) DNA sequence of the intronic *mdm2* promoter (P2). The Ets and AP-1 binding sites are indicated by arrows, the p53-responsive elements are boxed, and the TATA box is marked in bold.

(B) Various 5′ deletions of the *mdm2* promoter were cloned in front of the firefly luciferase gene. 10(1) cells were cotransfected with 1 μg of each luciferase reporter construct and either 100 ng of a vector expressing CAAX-tagged Raf or the parental control vector (pcDNA3). Luciferase activity was determined using a luminometer.

(C) Using in vitro mutagenesis, Ets and AP-1 sites of the *mdm2* promoter luciferase construct L1 as in (B) were selectively mutated. Luciferase activity of the mutated promoter constructs in 10(1) cells was determined. All promoter constructs were responsive to cotransfected p53 (indicated as +).

(D) 10(1) were transiently transfected with 1 μg of the *mdm2* promoter luciferase construct L1 combined with 100 ng of plasmids directing expression of either constitutively active MEK1, c-Ets-1, c-Ets-2, or c-jun. Luciferase activity was determined.

transcriptional activation in a transient assay. To assess the importance of the more downstream Ets (designated EtsB) and AP-1 sites in the *mdm2* P2 promoter in response to activated Raf, single point mutations were introduced into the full-length *mdm2* P2 promoter construct L1 by site-directed mutagenesis. All promoter constructs that contain mutations in one of the Ets sites or the AP-1 site are nonresponsive to activated Raf anymore (Figure 2C). Furthermore, combinations of mutations of the Ets and AP-1 sites led to a significant decrease of basal *mdm2* P2 promoter activity (Figure 2C). To exclude the occurrence of potential nonspecific mutations during the generation of these constructs and to address whether the Raf kinase and p53 responsive elements are distinguishable, p53 was cotransfected with the mutated P2 promoter constructs in parallel experiments. All mutated *mdm2* promoter constructs showed a strong induction by p53 (~10-fold) similar to that of the parental L1 mdm2 promoter construct, demonstrating a clearly distinct regulation of the P2 promoter by either Raf or p53 (data not shown). Overexpression of constitutively activated forms of MEK1 and MEK2, c-Ets-1, c-Ets-2, and c-jun, which are known downstream targets of Ras and Raf, led to an approximately 5- to 8-fold induction of *mdm2* P2 promoter activity, respectively (Figure 2D). To determine whether the *mdm2* AP-1 and Ets sites were capable of binding the respective transcription factors, nuclear extracts were prepared from NIH-3T3 cells expressing ΔB-Raf:ER* at various times after stimulation with 10 nM 4-HT. The *mdm2* AP-1 site in probe GS1 bound a complex in NIH-3T3 nuclear extracts, the formation of which was increased strongly after activation of Raf (Figure 3A). This complex was competed by 50-fold molar excess cold cognate competitor oligonucleotide or unlabeled GS1 probe but not by mutant AP-1 sequences or unlabeled probe GS2 (Figure 3A). When the labeled upstream *mdm2* Ets site (EtsA) was used as probe in electrophoretic mobility shift assays, formation of a complex could be detected, whose intensity remained unchanged after Raf activation. These studies demonstrate that transcription factors bind to the AP-1 and Ets elements in the *mdm2* promoter.

Raf Regulates p53 Levels through Its Effects on Mdm2

Induction of Mdm2 protein expression is expected to decrease levels of p53, since Mdm2 effectively promotes p53 degradation (Haupt et al., 1997; Kubbutat et al., 1997). To test this prediction, Raf kinase was activated in human tumor cells expressing mutant p53, and levels of Mdm2 and p53 proteins were analyzed by Western blotting. DKO4 cells were used, in which mutant

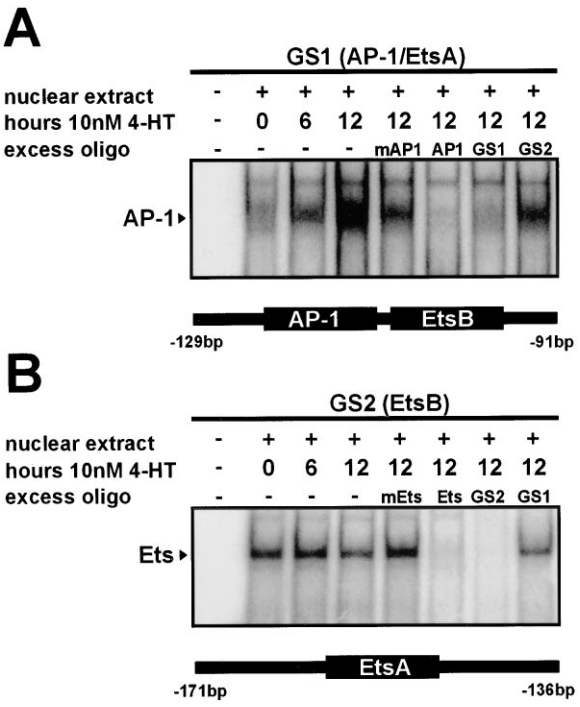

Figure 3. Transcription Factors Bind to the AP-1 and Ets Elements in the *mdm2* Promoter

(A) Nuclear extracts were prepared from NIH-3T3 cells expressing ΔB-Raf:ER* at the indicated time points after stimulation with 4-HT. EMSAs were performed with ^{32}P-labeled GS1-oligonucleotide comprising the AP-1/Ets element. In competition experiments, 50-fold molar excess of cold AP-1 or mutated AP-1 consensus oligonucleotide (AP1 and mAP1, respectively) was added to the binding reaction.
(B) EMSAs were carried out with ^{32}P-labeled GS2-oligonucleotide comprising the Ets site of the *mdm2* promoter.

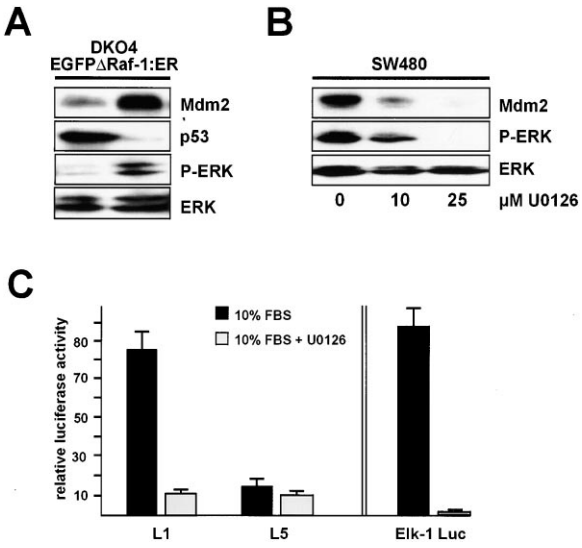

Figure 4. The Ras/Raf/MEK/MAPK Pathway Has an Impact on Mdm2 Expression Levels in Colon Cancer

(A) DKO4 cells expressing a EGFP-ΔRaf-1:ER construct were treated with 100 nM 4-HT. Twenty-four hours later, total cellular lysates were prepared. Equal amounts of proteins were separated by SDS-PAGE followed by immunoblotting with antibodies against Mdm2, p53, phosphorylated ERK1/2, or total ERK.
(B) SW480 cells were cultured 48 hr in the presence or absence of the MEK inhibitor U0126 and 10% fetal bovine serum. Equal amounts of proteins were electrophoretically separated prior to immunoblotting with Mdm2, phosphorylated ERK1/2, or total ERK antisera.
(C) SW480 cells were transfected with either 1 μg of the *mdm2* promoter luciferase construct L1 or the further 5′-deleted promoter construct L5, which lacks the Ras responsive elements. Transfected cells were incubated 48 hr in the presence or absence of 25 μM U0126 prior to measurement of luciferase activity. To monitor efficacy of the MEK inhibitor, U0126 Elk-1 activity was determined using a commercially available Elk-1 luciferase reporter system (right panel).

Ras had been deleted by homologous recombination (Shirasawa et al., 1993), and a conditionally active Raf allele (EGFP-ΔRaf-1:ER) was stably expressed in these cells (Woods et al., 1997). Raf kinase was turned on by addition of 4-HT, and Mdm2 levels accumulated (Figure 4A). p53 is transcriptionally inactive in these cells (Esteller et al., 2000). Hence, these data further confirm that Raf-dependent *mdm2* transcription is independent of p53 transcriptional activity. Importantly, increased Raf activity led to a dramatic decrease of p53 protein levels (Figure 4A). It is important to note that DKO4 cells, despite harboring a mutated p53 gene, do not express p14^{ARF} due to hypermethylation of the p14^{ARF} promoter (Esteller et al., 2000).

SW480 cells express an activated Ras allele and a mutant form of p53 that is transcriptionally inactive (Sharma et al., 1993). Treatment of these cells with the MEK inhibitor U0126 resulted in dose-dependent decrease in Mdm2 protein expression (Figure 4B) consistent with reduced MAPK activation, showing that sustained activity of the Ras/Raf/MEK/MAP kinase pathway is necessary for high levels of Mdm2.

To examine whether decrease of Mdm2 protein expression results from reduced transcriptional activity of the *mdm2* promoter upon MAPK inhibition, we measured *mdm2* P2 promoter activity. SW480 cells were transfected with either the *mdm2* P2 promoter construct L1, containing all the Raf-responsive elements, or the 5′ deleted L5 *mdm2* P2 promoter construct lacking the Ets and AP-1 binding motifs. Immediately after transfection, one fraction of the cells was incubated with 25 μM U0126 for 48 hr, while the other fraction of cells was incubated in medium without the MEK inhibitor. As displayed in figure 4C, an approximately 7-fold decrease of *mdm2* P2 promoter activity was observed upon U0126 treatment only in cells transfected with the Raf responsive *mdm2* promoter construct L1. Luciferase activity of cells containing the *mdm2* promoter construct L5 was significantly lower and did not change upon addition of MEK inhibitor U0126.

Ras Mutations Lead to Attenuation of the p53 Response and Increased Survival Rate upon γ-Irradiation

To investigate whether the increased Mdm2 protein levels due to constitutive activation of Ras can prevent or attenuate p53 induction in response to cellular stress, we γ-irradiated NIH-3T3 cells or NIH-3T3 cells stably expressing a vector encoding H-Ras (G12V). Figure 5A shows the time course of p53 induction in both cell lines. In the parental NIH-3T3 cells, p53 protein was dramatically induced after γ-irradiation. Increased p53 levels are already detectable after 30 min and reach a

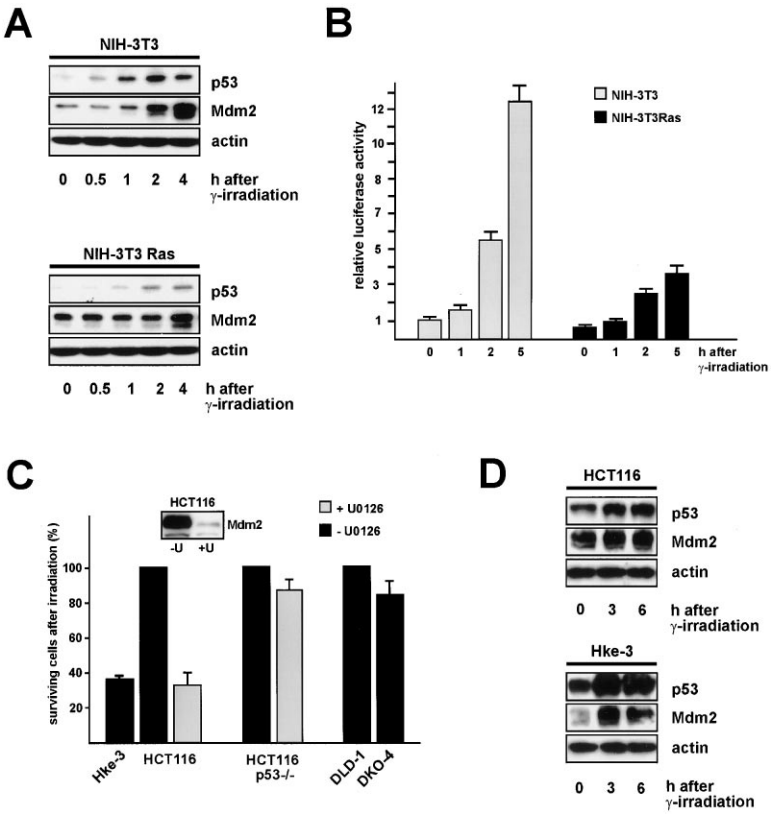

Figure 5. Kinetics of p53 Induction upon γ-Irradiation and Radiation Survival Are Affected by the Ras/Raf/MEK/MAPK Pathway

(A) Logarithmically growing cultures of NIH-3T3 cells stably transfected with oncogenic H-Ras(G12V) or vector alone were subjected to γ-irradiation at a dosage of 3 Gy. Proteins were electrophoretically separated, immobilized, and immunoblotted with antibodies specific to Mdm2, p53, or β-actin. Lysates (NIH-3T3 and NIH-3T3 Ras) were run on the same gel. Exposure time is identical for each protein shown, allowing direct comparison of signal intensity.

(B) 1×10^5 NIH-3T3 cells and Ras-transformed NIH-3T3 cells, respectively, were seeded in 6-well plates. The next day, cells were transfected with 1 μg p53-responsive luciferase reporter and exposed to ionizing radiation at a dosage of 3 Gy. Luciferase activity was determined using a luminometer.

(C) Clonal survival assays were performed using the human colon cancer cell lines HCT116 and DLD-1, and their isogenic derivatives Hke3, HCT116 $p53^{-/-}$, and DKO4, respectively. Cells were irradiated at a dosage of 5 Gy in a ^{137}Cs source. Survival rate for HCT116, HCT116 $p53^{-/-}$, and DLD-1, respectively, was set 100% in each set of comparisons.

(D) Time course of Mdm2 and p53 protein levels in HCT116 cells and its derivative Hke-3 after γ-irradiation (3 Gy). All lysates were run on the same gel. Exposure time is identical for each protein shown, allowing direct comparison of signal intensity.

maximum at 2 hr post irradiation. After 4 hr, p53 protein levels decreased but were still above the basal level in nonirradiated cells. Expression of Mdm2 protein in NIH-3T3 cells was strongly induced beginning 1 hr after irradiation of the cells and peaked at 4 hr. In contrast, γ-irradiation of NIH-3T3 cells harboring an activated Ras led to only a moderate increase of p53 protein. Furthermore, the onset of p53 induction in these cells is delayed compared to the parental NIH-3T3 cell line (1 hr in Ras-transformed NIH-3T3 cells versus 30 min in NIH-3T3 cells). While Ras-transformed NIH-3T3 cells have already higher basal Mdm2 protein levels (originating from the induced transcription of the *mdm2* P2 promoter by activated Ras), there was no significant increase of Mdm2 protein up to 4 hr after γ-irradiation. Thus, NIH-3T3 cells harboring an activated form of Ras are capable of attenuating the p53 response upon γ-irradiation, which is consistent with their increased basal Mdm2 protein expression.

Next, we wished to investigate the activity of the induced p53 in response to irradiation. We examined p53 activity after γ-irradiation in NIH-3T3 Ras cells using a luciferase assay. For this purpose, we transfected a synthetic p53 luciferase reporter construct into NIH-3T3 and Ras-transformed NIH-3T3 cells. Twenty-four hours after transfection, cells were irradiated at a dosage of 3 Gy and luciferase activity was determined at the indicated time points. Figure 5B displays the time course of p53 activation after γ-irradiation. While NIH-3T3 cells reveal a 12-fold induction of p53 transcriptional activity 4 hr after irradiation, their Ras-transformed counterparts show only a modest p53 response (4-fold). Thus, Ras overexpression attenuates not only p53 accumulation but also p53 activation in response to DNA damage.

This attenuation of p53 might explain the higher resistance of transformed NIH-3T3 cells toward γ-irradiation as determined in clonal survival assays (data not shown) (Sklar, 1988). Because Ras activation targets many downstream effectors (Katz and McCormick, 1997), we examined whether increased Mdm2 protein levels could mimick Ras-mediated radioresistance. Therefore, we established a stable NIH-3T3 cell line expressing Mdm2 protein under control of a mifepristone-inducible promoter (NIH-3T3-imdm2). Mifepristone-treated NIH-3T3-imdm2 cells and their parental counterpart, stably expressing the empty vector, were irradiated and subsequently, clonal survival assays were performed. Mdm2 expression partially rescues NIH-3T3 cells from irradiation-induced apoptosis (92% survivors in Mdm2 expressing NIH-3T3 versus 78% at 3 Gy; 49% versus 29% at 5 Gy).

To determine whether oncogenic Ras mutations confer radioresistance in human tumor cells, we performed clonal survival assays with the human colon cancer cell line HCT116 (wild-type p53, mutant Ras) and its derivative Hke-3, in which the mutant Ras has been deleted by homologous recombination (Shirasawa et al., 1993). Figure 5 shows Hke-3 express less Mdm2 protein and more p53 than HCT116 cells, consistent with a role for Ras in this pathway. p53 accumulated to significantly higher levels in Hke3 cells after γ-irradiation, presumably because of lower levels of Mdm2 (Figure 5D), and radiosensitivity of these cells increased dramatically (80% survivors versus 28% survivors at 5 Gy; similar results were obtained at a dosage of 1 Gy). Furthermore, inhibition of MEK in the parental HCT116 cell line through addition of U0126 resulted in a similar radiosensitive phenotype as in Hke-3 cells (Figure 5D). Treatment of

the p53-deficient isogenic cell line HCT116 $p53^{-/-}$ (Bunz et al., 1998) with the MEK inhibitor U0126 revealed no significant difference in radiosensitvity, showing that Ras-mediated radioresistance is dependent on p53. The colon cancer cell line DLD-1 harboring mutant p53 and Ki-Ras genes shows no difference in radioresistance compared to its counterpart DKO4, in which the mutant Ras has been deleted (Figure 5C), suggesting that Ras has no impact in cells lacking p53. However, it is important to note that DLD-1 and HCT116 cells do not share an identical genetic background. Nonetheless, this demonstrates an important role for oncogenic Ras mutations in the presence of wild-type p53 in conferring radioresistance in human tumors.

Taken together, these results provide strong evidence that activated H-Ras leads to increased basal Mdm2 protein expression, which is capable of attenuating p53 induction upon γ-irradiation and renders cells more resistant to the inhibitory effects of irradiation.

Attenuation of p53 Accumulation in Response to DNA-Damaging Reagents by Activated Raf Is Dependent on the $p19^{ARF}$ Status

$p19^{ARF}$ physically interacts with Mdm2 and consequently stabilizes p53 (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998). To investigate whether the presence of $p19^{ARF}$ can prevent Raf-induced Mdm2 from degrading p53, we established wild-type MEFs and $p19^{ARF}$ null MEFs expressing a conditionally active Raf allele (EGFP-ΔRaf-1:ER) (Kamijo et al., 1997). MEFs and $p19^{ARF}$ null MEFs with induced and noninduced Raf kinase were treated with the DNA damaging reagent adriamycin (0.25 μg/ml). As shown in Figure 6, activation of Raf leads to a significant induction of Mdm2 protein after 24 hr in both cell lines. Concomitantly, $p19^{ARF}$ levels increase in 4-HT-treated wild-type MEFs expressing EGFP-ΔRaf1:ER (Figure 6A). While Raf-induced Mdm2 can attenuate p53 accumulation in response to DNA damage in $p19^{ARF}$ null MEFs, we detect no attenuation of p53 response in wild-type MEFs upon Raf activation (Figures 6A and 6B). These results provide evidence that $p19^{ARF}$ is capable of neutralizing the increased Mdm2 protein in response to activation of the Ras/Raf/MEK/MAP kinase pathway. If $p19^{ARF}$ is not expressed due to a targeted deletion of exon1β of the INK4A gene locus (Kamijo et al., 1997), induced Mdm2 protein is fully functional, can bind to p53, and promote its degradation.

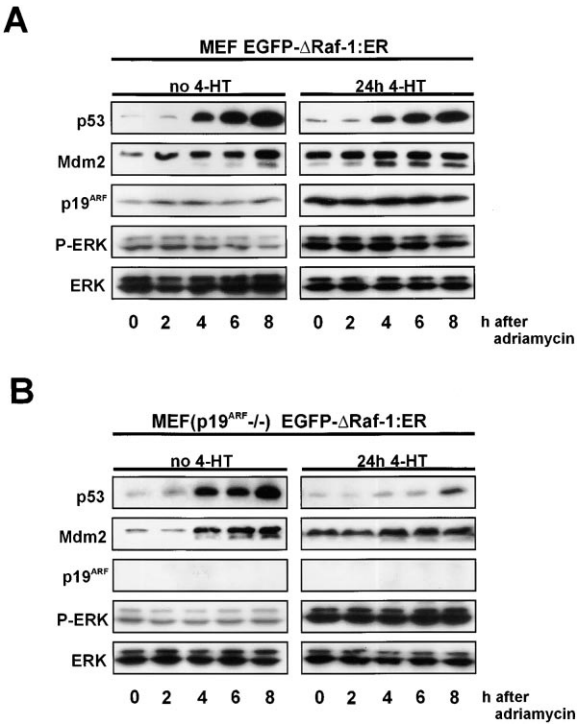

Figure 6. Attenuation of the p53 Accumulation by Raf-induced Mdm2 in Response to DNA Damage Depends on the $p19^{ARF}$ Status

(A) Wild-type MEFs expressing a 4-Hydroxy-Tamoxifen (4-HT) inducible EGFP-ΔRaf1:ER construct were treated with 1 μM 4-HT (right panel), or mock treated (left panel). After 24 hr, adriamycin (0.25 μg/ml) was added to the culture medium. Total protein was harvested at the indicated time points after addition of adriamycin and subjected to Western blot analysis. Lysates (+/− 4-HT) were run on the same gel. Exposure time is identical for each protein shown, allowing direct comparison of signal intensity.

(B) $p19^{ARF}$ null MEFs expressing a 4-Hydroxy-Tamoxifen (4-HT) inducible EGFP-ΔRaf1:ER construct were treated with 1 μM 4-HT (right panel), or mock treated (left panel). Twenty-four hours later, adriamycin (0.25 μg/ml) was added to the medium.

Discussion

p53 is the major known regulator of its own inhibitor, Mdm2 (Barak et al., 1993; Wu et al., 1993). Mdm2-mediated degradation of p53 occurs in the cytoplasm, through a proteasome-dependent pathway (Haupt et al., 1997; Kubbutat et al., 1997). Hence, p53 and Mdm2 have been postulated to form an autoregulatory negative feedback loop. Regulation of *mdm2* expression by p53 is thought to keep p53 function under control, thereby preventing widespread p53-dependent apoptosis (Lane and Hall, 1997; Prives, 1998).

In this study, we demonstrate that Mdm2 expression is also modulated by the Ras/Raf/MEK/MAP kinase pathway through activation of Ets and AP-1 sites in the P2 promoter, upstream from the p53 responsive element and independent of its activity. Furthermore, Mdm2 induced by the Ras/Raf/MEK/MAP kinase pathway is functionally active and leads to degradation of p53. This signaling pathway is intact in tumor cells expressing activated Ras as Mdm2 protein levels decrease dramatically after inhibiting MEK activity in these cells. Importantly, the effects of induced Mdm2 on p53 are regulated by $p19^{ARF}$. Ras therefore acts on p53 through two competing pathways (Figure 7). Activation of the Ras/Raf/MEK/MAP kinase cascade results in elevated levels of Mdm2 protein. However, in normal cells, this pathway also induces the expression of $p19^{ARF}$ (Bates et al., 1998; Palmero et al., 1998), which inhibits Mdm2 activity (Tao and Levine, 1999; Sherr and Weber, 2000). Thus, in normal cells, levels of p53 are determined by a balance between opposing effects of the Ras/Raf/MEK/MAP kinase pathway. In MEFs, these opposing effects are equivalent, and Raf is ineffective at inducing p53, despite its effects in $p19^{ARF}$. In different cell types, or even in MEFs growing under slightly different conditions, the balance of these opposing pathways is likely to be different. For example, in IMR90 human diploid fibroblasts, activated MEK leads to accumulation of p53, presumably because $p14^{ARF}$ exceeds Mdm2 induction (Lin et al., 1998). In Figure 7, DNA damaging agents are shown

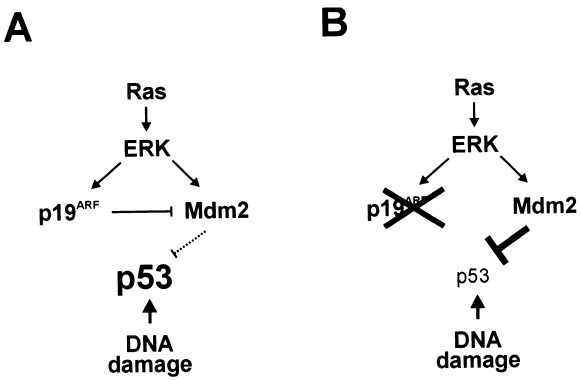

Figure 7. Model for the Regulation of p53 by the Ras/Raf/MEK/MAP Kinase Pathway

(A) Control of p53 levels in wild-type cells. Activation of the Ras/Raf/MEK/MAP kinase leads to transcriptional induction of $p19^{ARF}$ by E2F-1 via the cyclin D/CDK4/Rb pathway. Although Mdm2 protein is also induced, $p19^{ARF}$ is capable of keeping Mdm2 functionally inactive. p53 accumulates rapidly after DNA damage.

(B) Control of p53 levels in cells lacking $p19^{ARF}$. Activated Ras/Raf/ERK kinase induces the transcription of *mdm2*. Because $p19^{ARF}$ is not expressed, the elevated Mdm2 protein is functionally active and attenuates p53 accumulation in response to DNA damage.

regulating p53 in a $p19^{ARF}$-independent manner; however, it is possible that $p19^{ARF}$ affects the magnitude or duration of the p53 response to DNA under some physiological circumstances. In contrast to the Ras/Raf/MEK/MAP kinase pathway, which activates opposing regulators of p53, E1A induces $p19^{ARF}$ but does not directly induce Mdm2 (de Stanchina et al., 1998). E1A is therefore a potent inducer of p53.

p53 function is lost by mutation of the p53 gene in about 60% of all human tumors (Hollstein et al., 1991; Levine et al., 1991). In many of the remaining tumors, p53 function is abrogated by overexpression of Mdm2 (Oliner et al., 1992), expression of HPV E6 (Scheffner et al., 1991), or by loss of $p14^{ARF}$ expression (Kamb et al., 1994; Cordon-Cardo, 1995; Haber, 1997). In about 30% of human tumors, Ras is activated by mutation (Bos, 1989). Although Ras mutation and p53 mutation seem to be independent events (Mitsudomi et al., 1992), Ras-induced Mdm2 might block p53 from inducing apoptosis or growth arrest in the early phase of tumor development allowing coexistence of Ras mutations and wild-type p53. For example, Ras mutations precede p53 mutations in the stepwise development of colon cancer (Kinzler and Vogelstein, 1996). Activation of Ras may suppress p53 during the early stages of tumor development. Moreover, some mutant forms of p53 may retain residual activity (Friedlander et al., 1996). Elevated Mdm2 protein levels induced by Ras activation may bind those mutated forms of p53 and abolish remaining p53 function. Consistent with this, we find that the Ras/Raf/MEK/MAP kinase pathway suppresses expression of mutant p53 in DKO4 colon cancer cells. In addition, p53-independent transformation properties of Mdm2 have been reported. Sarcomas harboring *mdm2* gene amplifications together with p53 mutations show worse prognosis, when compared to tumors with genetic alterations of p53 or *mdm2* alone (Cordon-Cardo et al., 1994). Therefore, Ras-induced Mdm2 might contribute to tumor progression in a p53-independent manner. Mouse models have revealed that tumors in which p53 is lost through mutation or by loss of $p19^{ARF}$ are phenotypically similar, at least in the early stages of tumor development (Donehower et al., 1992; Kamijo et al., 1997). However, tumors that have lost p53 by direct mutation are genetically unstable relative to those that have lost p53 function through mutation of $p19^{ARF}$ (Kamijo et al., 1997). The latter tumors may retain residual p53 function that protects cells from genetic rearrangement. Indeed, p53 transcriptional activity can be measured easily in $p14^{ARF}$-deficient tumor cells that retain low levels of p53 protein. In these tumors, activation of Ras may suppress residual p53 and allow tumor progression.

In addition to a role in tumor development, Ras regulation of *mdm2* and p53 may have important implications in cancer treatment. Tumors carrying constitutively active forms of Ras might be more resistant to treatment with ionizing radiation and chemotherapy. This idea is supported by previous observations that Ras-transformed NIH-3T3 cells are relatively resistant to radiation induced apoptosis (FitzGerald et al., 1985; Sklar, 1988). Chang and coworkers examined the radiation survival of various NIH-3T3 transformants representative of the various classes of oncogenes that may be involved in the pathway. In their study, Ras, Raf, Ets, and jun overexpressed in NIH-3T3 cells conferred a radiation resistant phenotype (Pirollo et al., 1993). Interestingly, chronic Ras transformation leads to an increase in AP-1 activity and upregulation of c-jun (Cook et al., 1999). As overexpression of transcription factors c-jun and Ets mediates radioresistance, a newly transcribed protein that functions as an inhibitor of radiation-induced apoptosis is likely to be involved. We provide evidence that Mdm2, a well characterized inhibitor of p53 activity, mediates the radioresistant phenotype conferred by oncogenic Ras. Our study demonstrates that constitutively active Ras induces *mdm2* transcription via activation of transcription factors binding to the AP-1 and Ets elements within the promoter. In the absence of $p19^{ARF}$, elevated Mdm2 protein levels resulting from constitutively active Ras/Raf/MEK/MAP signaling lead to attenuated and diminished p53 response and increased survival rates upon DNA damage. We propose that higher basal levels of Mdm2 protein in Ras-transformed cells prevents the accumulation of stable p53 protein and the subsequent induction of apoptosis or growth arrest in response to DNA damage.

Recently, we have suggested that high levels of Mdm2 activity explain why the E1B55k-deleted adenovirus dl1520 (ONYX-015) replicates efficiently in many tumor cell lines that retain wild-type p53 (Ries et al., 2000). Oncogenic Ras, or other events that upregulate the MAP kinase pathway, could contribute to replication of dl1520 in these cells by elevating levels of Mdm2 protein.

In vivo studies provide further evidence for the importance of Ras-controlled Mdm2 expression. Inhibition of oncogenic Ras activity in mouse models, through pharmacological intervention or genetic manipulation, leads to death by apoptosis (Lebowitz et al., 1997; Johnson et al., 1997; Heimbrook and Oliff, 1998). This could be due, in part, to activation of p53 through reduced expression of Mdm2. To reevaluate the role of *mdm2* and $p19^{ARF}$ in Ras-induced tumor development in vivo, we are currently investigating the contribution of this pathway in a defined multistage mouse tumor model.

In conclusion, we have shown that Mdm2 is a transcriptional target of the Ras/Raf/MEK/MAP kinase pathway, and that this activation is independent of p53. Ras

therefore regulates p53 through opposing pathways involving Mdm2 and its inhibitor $p14^{ARF}$. In cancer cells lacking $p14^{ARF}$, Ras suppresses p53 expression. This may have important implications in cancer development and therapy.

Experimental Procedures

Antibodies and Reagents

Rabbit polyclonal anti-phospho-ERK and anti-ERK antibodies were obtained from NEB, mouse monoclonal anti-β-actin antibody was purchased from Sigma, and sheep polyclonal anti-p53 antibody Ab-7 was obtained from Calbiochem. Mouse monoclonal anti-Mdm2 antibody 2A10 was kindly provided by G. Zambetti (St. Jude Children's Hospital). The Dual Luciferase system and the MEK-inhibitor U0126 were from Promega.

Plasmids

Retroviral vectors (pBabe puro or pWZL3blast) expressing ΔB-Raf:ER*, EGFPΔRaf-1:ER proteins, or H-Ras(G12V) have been described elsewhere (Rodriguez-Viciana et al., 1997; Woods et al., 1997). Antisense riboprobe for RNase protection assay contains the murine *mdm2* cDNA fragment spanning from nt +264 to nt +3 (Barak et al., 1994).

Tissue Culture and Cell Lines

$p19^{ARF}$ null mouse embryo fibroblasts (Kamijo et al., 1997) and wild-type mouse embryo fibroblasts expressing the EGFP-ΔRaf-1:ER construct were used for experiments between passage 5 and 8 (Kamijo et al., 1997).

All cell lines were maintained in phenol-free DMEM H21 medium to prevent basal activation of the Raf:ER fusion protein. Asynchronous cell populations were treated with 10 nM 4-HT (for ΔB-Raf:ER*) or 100 nM (for EGFPΔRaf-1:ER) at 80% confluency for 24 hr or 48 hr, respectively. SW480 cells were maintained in Leibovitz medium supplemented with 10% heat-inactivated fetal bovine serum. To establish an inducible Mdm2 NIH-3T3 cell line, the commercially available pSwitch system (Invitrogen) was used. In radiation survival experiments, NIH-3T3 cells stably transfected with the pSwitch vector only treated with mifepristone (10^{-8} M) served as control.

Transfections and Luciferase Reporter Gene Assay

Reporter assays were performed as described previously using the Dual Luciferase system from Promega (Biederer et al., 2000). p53 activity was monitored using the PathDetect In Vivo Signal Transduction Pathway *Cis*-Reporting System (Stratagene, LaJolla, CA).

Northern Blot Analysis

Total cellular RNA was isolated using the RNeasy kit (Qiagen). Ten micrograms of total RNA per lane was subjected to a 0.9% agarose formaldehyde gel electrophoresis, transferred to Hybond N^+ membrane, and UV cross-linked (1200 μJ). Prehybridization, hybridization, and washing of blots were performed at 60°C in QuickHyb buffer (Amersham). The blots were stripped by boiling in 0.1% SDS, and then reprobed.

RNase Protection Assay

Ribonuclease protection assays were performed using the RPA II kit (Ambion) and a cDNA riboprobe corresponding to exons 1–3 of the full-length *mdm2* transcript. *Mdm2* mRNA arising from the upstream promoter protects a fragment of 260 nt, whereas mRNA initiated from the internal promoter (P2) yields a fragment of 147 nt (Barak et al., 1994).

Western Blot Analysis

Cells were lysed in 2×Laemmli buffer and equal amounts of protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with various antibodies. All immunoblots were visualized by enhanced chemiluminescence detection system (Amersham).

Site-Directed Mutagenesis

Missense mutations were engineered into the various mdm2 promoter luciferase constructs by primer-mediated mutagenesis using the QuickChange mutagenesis kit (Stratagene). All mutations were verified by sequencing.

Electrophoretic Mobility Shift Assay

Double-stranded oligonucleotides spanning regions −129 bp to −91 bp (GS1), and −171 bp to −136 bp (GS2) of *mdm2* intron 1 (respective to the first nucleotide of exon 2) were labeled with [α-^{32}P]dCTP. Nuclear extracts of NIH-3T3 cells were prepared as described elsewhere (Andrews and Faller, 1991). EMSAs were carried out using 10 μg nuclear extract as previously described (Moser et al., 1995).

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